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**NEW DATA ON TAXONOMY AND DISTRIBUTION OF *KALUGINIA*
LEBETIFORMIS MAKARCHENKO, 1987 (DIPTERA: CHIRONOMIDAE,
DIAMESINAE) FROM EAST ASIA**

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Summary. In comparing the results of DNA barcoding of adult males of *Kaluginia lebetiformis* Makarchenko from South Korea and the Amur River basin of the Russian Far East, it turned out that the K2P genetic distance obtained using the site of cytochrome oxidase 1 mtDNA 658 bp long between the individuals of both studied populations is 0.641 and corresponds to the species level. However, we did not reveal sufficient morphological differences of the “Korean” and “Amur” chironomids to establish independent species and found it appropriate to give subspecies status for these populations, namely *Kaluginia lebetiformis lebetiformis* Makarchenko for specimens from the Russian Far East and *K. lebetiformis koreana* **subsp. n.** for specimens from South Korea. Illustrated descriptions of adult males of both subspecies as well as the results of DNA barcoding of *K. lebetiformis lebetiformis* from Amur River basin in comparison with *K. lebetiformis koreana* **subsp. n.** and some other known taxa of the tribe Boreoheptagyini are provided.

Key words: Diptera, Chironomidae, Diamesinae, *Kaluginia*, taxonomy, DNA barcoding, East Asia.

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Резюме. При сравнении результатов ДНК-баркодирования имаго самцов *Kaluginia lebetiformis* Makarchenko из Южной Кореи и бассейна р. Амур российского Дальнего Востока оказалось, что генетическая дистанция, полученная с использованием участка цитохромоксидазы 1 мтДНК длиной 658 пн. между особями этих двух популяций, составляет 0,641 и соответствует видовому уровню. Однако, мы не выявили достаточных морфологических различий в строении «корейских» и «амурских» комаров для выделения самостоятельных видов, но нашли целесообразным установить для этих популяций

подвидовой статус – *Kaluginia lebetiformis lebetiformis* Makarchenko для особей с российского Дальнего Востока и *K. lebetiformis koreana* **subsp. n.** для экземпляров из Южной Кореи. В работе приведены иллюстрированные описания имаго самцов обоих подвидов с замечаниями по таксономии и распространению, а также результаты ДНК-баркодирования *K. lebetiformis lebetiformis* из басс. р. Амур в сравнении с данными по *K. lebetiformis koreana* **subsp. n.** и другим таксонам трибы Boreoheptagyini.

INTRODUCTION

The genus *Kaluginia* Makarchenko, 1987 was established with the description of *K. lebetiformis* Makarchenko, 1987 by two adult males from the south part of Sakhalin Island (Makarchenko, 1987). Later one male in bad condition was found in Khasansk District of Primorye Territory of the Russian Far East and four males were collected in South Korea (Makarchenko *et al.*, 2017, 2018), what made possible to rewrite the male according to Korean material and get the results of DNA barcoding. Additional material collected in 2019 by N. M. Yavorskaya in the Amur River basin also was used for DNA barcoding and comparison of the received data with those of the South Korean population. A reference 658 bp barcode sequence from a fragment of the mitochondrial gene cytochrome oxidase I (COI) was used as a tool for species delimitation. K2P genetic distance between both studied populations was 0.641. This value is appropriate threshold for species delimitation but we haven't revealed significant morphological diagnostic features for the description of two species and therefore, we found it appropriate to distinguish these populations as subspecies – *Kaluginia lebetiformis lebetiformis* Makarchenko for specimens from the Russian Far East and *K. lebetiformis koreana* **subsp. n.** for specimens from South Korea.

Below we are provided illustrated descriptions of adult males of both subspecies as well as the results of DNA barcoding of *K. lebetiformis lebetiformis* from Amur River basin in comparison with known taxa of the tribe Boreoheptagyini. We also have evaluated a fragment of mitochondrial Cytochrome Oxidase I (COI), which represents the barcode region, as described by Hebert *et al.* (2003, 2004). Standardized mitochondrial fragments from COI (DNA barcodes) have been used to identification non-biting mites of subfamily Diamesinae (Montagna *et al.*, 2016; Makarchenko *et al.*, 2018, Makarchenko & Semchenko, 2014). K2P genetic distances between DNA barcodes of the genus *Kaluginia* also have been used to evaluate intersubspecific variability. Moreover, we have constructed the Bayesian tree using obtained DNA barcodes and some other species of the tribe from GenBank.

MATERIAL AND METHODS

The material was preserved in 96% ethanol for DNA-analysis and in 70% ethanol for further study of morphology, and slide-mounted following the methods by Makarchenko (1985). The terminology follows Sæther (1980). Holotype and paratypes of a new subspecies as well as other material are deposited in the Laboratory of Freshwater Hydrobiology of the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok.

For barcoding the material was deposited in refrigerator (+4°C). Total DNA was extracted from each sample using the Invitrogen PureLink Genomic DNA Mini Kit in accordance with the protocol in a final elution volume of 80 µL. A 650–700 bp fragment of the COI barcode region was amplified with the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') described in Folmer *et al.* (1994). PCR amplification was carried out in a final volume of 10 µL, including 5 µL of

Go Taq Green Master Mix (Promega), 0.5 µL of each primer (100 ng/µl), 3 µl nuclease-free water and 1 µL of purified DNA. All PCR amplifications were verified using electrophoresis on a 1,5% TBE agarose by visualizing on GelDoc XR+ imaging systems (Bio-Rad). Two positive PCR products were purified for cycle sequencing using Exonuclease I (ExoI) and Thermosensitive Alkaline Phosphatase (FastAP) by ThermoFisher Scientific. DNA sequencing was performed on both strands using BigDye 3.1® sequencing kit (ThermoFisher Scientific), in a 10 µL reaction including 1.25 µL ABI 5X dilution buffer, 1 µL Big Dye, 0.5 µL of primer, 0.8 µL of PCR product and 6.45 µL nuclease free water. The sequencing amplification protocol consisted of one cycle of 1 min at 98°C, followed by 30 cycles of 10 sec at 96°C, 5 sec at 50°C, and 4 min at 60°C. Each sequencing reaction was purified by D-pure Dye Terminator (Nimagen) according to the protocol. Finally, all samples were analyzed in an ABI 3130XL® (Applied Biosystems) automated capillary sequencer. Consensus sequences and interspecies genetic distances based on the K2P were obtained with MEGA 7 (Kumar *et al.*, 2016). The obtained sequences were checked aligned at the nucleotide level using MUSCLE (Edgar, 2004).

Bayesian phylogenetic analyses were conducted with MrBayes v. 3.2.7 (Ronquist & Huelssenbeck, 2003). PartitionFinder 2.1.1 (Lanfear *et al.*, 2012) was used to select the best-fit partitioning scheme and models separately for each codon position of COI gene using the greedy algorithm with linked branch lengths for the corrected Bayesian Information Criterion as the optimality criterion for model selection. The best model for 1 codon position was SYM + I (Zharkikh, 1994), for 2 codon position - F81 (Felsenstein, 1981) and for 3 codon position - HKY + G (Hasegawa *et al.*, 1985). Bayesian Inference was performed with two independent runs of Metropolis-coupled Markov chain Monte Carlo analyses. The chains were run for 5 million generations and sampled every 100 generations. A burn-in of 100,000 generations (or 5% of the sampled trees) was used. Moreover, trace files were visually inspected in Tracer 1.7 (Rambaut *et al.*, 2018). FigTree v. 1.4.4 was used to visualize phylogenetic trees after analysis. All sequences of *Kaluginia lebetiformis lebetiformis* Makarchenko and *K. lebetiformis koreana* **subsp. n.** have been deposited in GenBank (accession numbers respectively MN625616–MN625617 and MH547037–MH547038).

MORPHOLOGICAL DESCRIPTIONS

Kaluginia lebetiformis lebetiformis Makarchenko, 1987

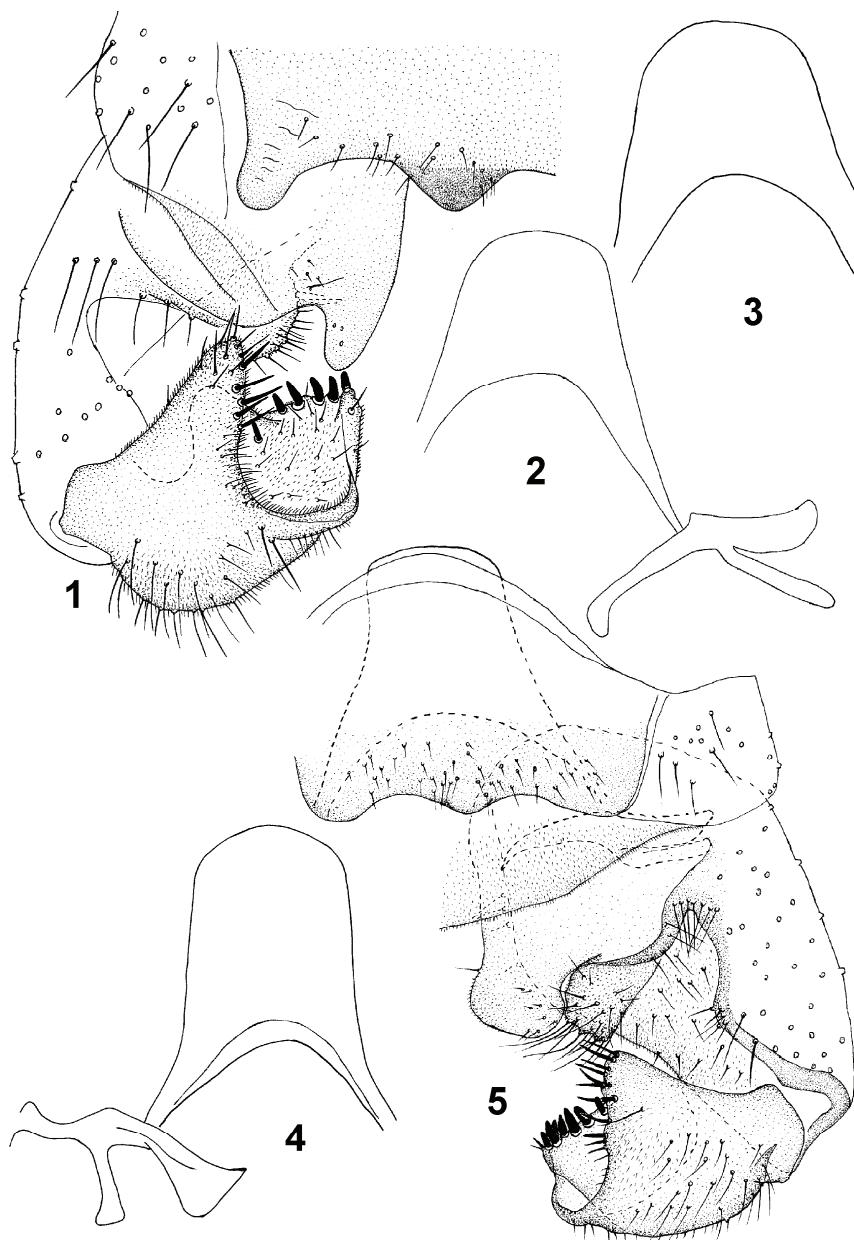
Figs 1–3, 6–8

Kaluginia lebetiformis Makarchenko, 1987: 786, 2006: 266; Oliver, 1989: 134; Ashe & Connor, 2009: 291; Makarchenko *et al.*, 2018: 28 (partly), Fig. 4.

MATERIAL EXAMINED. **Russia:** South Sakhalin, Dolinsk District, Sokol Village, Belaya River, 29.VI 1985, 1 ♂ (holotype), leg. S. Bestalannaya, E. Makarchenko; Khabarovsk Territory, Nanaisky District, Anyuisky National Park, Pihtsa River (tributary of Gassi Lake), Amur River basin, N 48.47.804, E 136.47.027, 22–24.V 2019, 4 ♂♂, leg. N. Yavorskaya; Primorye Territory, Khasansk Region, Barabashevka River, 14.V 2002, 1 ♂, leg. V. Teslenko.

DESCRIPTION. *Male imago* (n=4). Total length 3.2–3.7 mm. Total length/wing length 0.82–1.62. Total coloration brown to dark-brown; antepnotum light yellow; methonotum yellowish, with brown stripes; legs spotted: basal 2/3 of femur yellowish, distal 1/3 brown; tibia in basal and apical parts brown and yellowish in middle part; basal 2/3 of ta1 yellowish and distal 1/3 brown; ta₂–ta₅ brown.

Head. Temporal setae (from one side) including 2–4 frontals, 6–7 orbitals and 8–12 verticals. Clypeus with 12–21 setae. Palpomere length (µm): 36, 48–70, 92–105, 124–160, 144–248

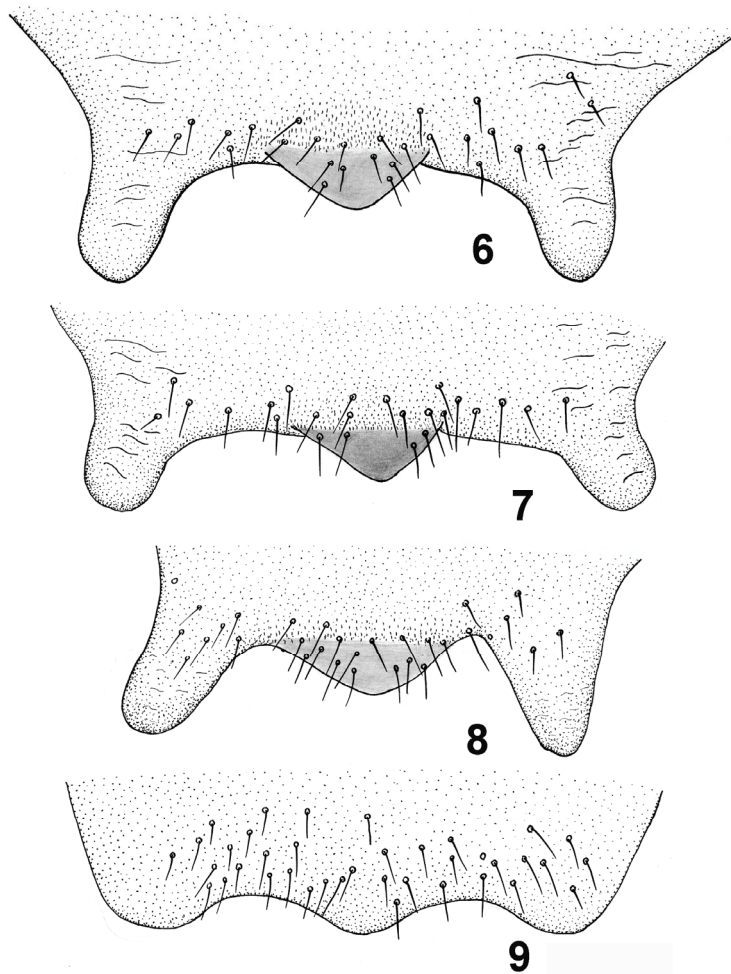


Figs 1–6. Adult males of *Kaluginia lebetiformis lebetiformis* Makarchenko (1–3) and *K. lebetiformis koreana* **subsp. n.** (4–5). 1, 5 – total view of hypopygium, from above; 2, 4 – transverse sternapodeme and aedeagal lobe; 3 – transverse sternapodeme. Scale bars – 50 μ m.

Head width/palpal length 0.84–1.11. Antenna with 7 flagellomeres and reduced plume of setae 35–75 μm long. Flagellomeres length (μm): 44–45, 24–48, 25–32, 25–28, 30–32, 32–33, 65–68; terminal flagellomere with 1–2 subapical setae 48–68 μm long; scape with 3 setae. AR 0.31–0.38.

Thorax. Antepronotum with 12–26 lateral setae 64–72 μm long. Acrostichals 17–31 (48–60 μm), dorsocentrals 18–26 (60–100 μm), prealars 17–27, supraalars 1–2, scutellars 73–78.

Wing. Length 2.28–4.20 mm, width 0.72–1.2 mm. Anal lobe well developed; squama with 22–36 setae in 1–2 rows. R and R_1 with 57–65 setae (32–40 μm), R_{4+5} with 20–31 setae (32–40 μm). R_{2+3} absent.



Figs 6–9. Posterior part of tergite IX of adult males *Kaluginia lebetiformis lebetiformis* Makarchenko from Amur River basin (6–7), South Sakhalin [holotype] (8) and *K. lebetiformis koreana* **subsp. n.** from South Korea (9). Scale bars – 50 μm .

Legs. BR₁ 2.1; BR₂ 1.5–2.0; BR₃ 2.1. Spur of front tibia 40–54 µm long. Spurs of middle tibia 50–60 and 45–52 µm long. Spurs of hind tibia 64–88 µm and 48–55 µm long. Hind tibial comb with 10–14 spine-like setae. Middle legs with 6 pseudospurs 32–40 µm long on ta₁ and with 8 pseudospurs on ta₃; hind legs with 4–5 pseudospurs on ta₁. Claw with 4 denticles apically. Length (µm) and proportions of leg segments are as follow:

P	fe	ti	ta ₁	ta ₂	ta ₃	ta ₄	ta ₅	LR	BV	SV
P ₁	983–1148	1197–1279	749–804	377–410	151–410	82–88	115–131	0.62–0.63	3.45–3.90	2.88–3.06
P ₂	1071–1230	1096–1246	623–787	328–394	189–213	66–82	113–131	0.53–0.64	3.65–4.06	3.06–3.77
P ₃	1071–1230	1260–1476	743–804	416–476	213–230	76–98	126–148	0.51–0.59	3.65–3.93	3.14–3.59

Hypopygium (Figs 1–3, 6–8). Tergite IX with round-triangular dark anal point, 24–28 µm long and 60 µm width, and with 25–33 setae, 12–24 µm long; anal-lateral corners of tergite IX elongated in form of narrow lobes back; laterosternite IX with 12–17 setae (from one side), 48–68 µm long. Transverse sternapodeme high, almost trapezoidal, 96–128 µm long, and 104–108 µm wide in basal part, with rounded apex. Gonocoxite 278–280 µm long. Basal lobe of gonocoxite is various shapes and depends on its position; in the inner half with microtrichia, along the outer margin with short setae; inferior volsella as in Fig. 1, covered by setae. Gonostylus 152–164 µm long, scoop-shaped; inner lobe along the margin with 5–6 megasetae 14–16 µm long and one subterminal tooth; outer lobe widely triangular, inner margin of which with strong setae 16–28 µm long. HR 1.74–1.79.

DISTRIBUTION. This subspecies is known from Sakhalin Island, Primorye Territory and Amur River basin of the Russian Far East.

***Kaluginia lebetiformis koreana* Makarchenko, subsp. n.**

<http://zoobank.org/NomenclaturalActs/3D03C35E-24AF-4831-BAAB-6C5029D563E4>

Figs 4–5, 9

Kaluginia lebetiformis Makarchenko, 1987; Makarchenko *et al.*, 2018: 28 (partly), Figs 1–3, 5–10.

MATERIAL EXAMINED. Holotype: 1♂, **South Korea**: Gyeonggi-do, Gapyeong-gun, Gapyeongcheon, Bukhan River, Han River basin, 15. IV 2016 (Light trap), 37°58' N, 36.4° 127°26'35.5" E, leg. Y. Bae. Paratypes: 1♂, the same data as holotype, leg. Y. Bae; 1♂, the same data as holotype, except 22.V 2014 (Light trap), leg. H. Kang.

DESCRIPTION. *Male imago* (n=3). Total length 2.3–2.6 mm. Total length/wing length 1.03–1.10. Total coloration brown to dark-brown; antepnotum light yellow; methonotum yellowish, with brown stripes; legs spotted: basal 2/3 of femur yellowish, distal 1/3 brown; tibia in basal and apical parts brown and yellowish in middle part; basal 2/3 of ta₁ yellowish and distal 1/3 brown; ta₂–ta₅ brown (Makarchenko *et al.*, 2018, Fig. 1).

Head. Temporal setae (from one side) including 3–4 frontals, 7 orbitals and 7–10 verticals. Clypeus with 17–26 setae. Palpomere length (µm): 28–48, 60–64, 104–120, 132–140, 212–220. Head width/palpal length 0.92–1.0. Antenna with 7 flagellomeres and reduced plume of setae 48–68 µm long (Makarchenko *et al.*, 2018, Fig. 2). Flagellomeres length (µm): 52–56, 24, 28, 24–28, 28–32, 28–32, 68–84; terminal flagellomere with 2 subapical setae 60–76 µm long; scape with 3 setae 28–36 µm long. AR 0.34–0.46.

Thorax. Antepronotum with 18–20 lateral setae. Acrostichals 25 (48–56 μm), dorso-centrals 21–27 (72–100 μm), prealars 16, supraalars 1, scutellars *ac* 50.

Wing. Length 2.08–2.52 mm, width 0.68–0.76 mm. Anal lobe well developed; squama with 19–31 setae (64–80 μm) in 1–2 rows. R and R₁ with 55–58 setae (32–36 μm), R₄₊₅ with 24–29 setae (28–40 μm) in subapical part. Wing of one male with 3 short setae on M₃₊₄.

Legs. BR₁ 2.0; BR₂ 1.8; BR₃ 1.9. Spur of front tibia 44 μm long. Spurs of middle tibia 28–52 and 44–53 μm long. Spurs of hind tibia 68–72 μm and 44–52 μm long. Hind tibial comb with 12–14 spine-like setae. Middle legs with 4 pseudospurs 36–40 μm long on ta₁; hind legs with 7 pseudospurs on ta₁. Claw with 4 denticles apically. Length (μm) and proportions of legs segments are as follow:

P	fe	ti	ta ₁	ta ₂	ta ₃	ta ₄	ta ₅	LR	BV	SV
P ₁	918–	1132–	722–	394–	197–	66–74	98–	0.64–	3.67–	2.76–
	1115	1328	886	459	213		115	0.67	3.87	2.84
P ₂	984–	1082–	574–	312–	189–	74–	115	0.53–	3.61–	3.31–
	1197	1246	738	394	213	115		0.59	4.0	3.60
P ₃	1000–	1230–	705–	394–	213–	66–74	98–	0.57–	3.69–	3.0–
	1214	1443	886	508	246		131	0.61	3.81	3.16

Hypopygium (Figs 4–5, 9). Tergite IX with anal point which like small rounded protuberance, and with 29–42 setae, 12–20 μm long; anal-lateral corners not or slightly elongated; laterosternite IX with 15–20 setae (from one side), 44–48 μm long. Transverse sternapodeme high, almost trapezoidal, 128–132 μm long and 148–152 μm wide in basal part, 88–100 μm wide in subapical part, with rounded apex. Gonocoxite 308–310 μm long. Basal lobe of gonocoxite is various shapes and depends on its position; in the inner half with microtrichia, along the outer margin with short setae; inferior volsella as in Fig. 5, covered by setae 40–60 μm long. Gonostylus 156 μm long, scoop-shaped; inner lobe along the margin with 5–9 megasetae 12–16 μm long and one subterminal tooth; outer lobe widely triangular, inner margin of which with strong setae 20–24 μm long, outer half with thinner and longer setae 56–72 μm long. HR 1.97.

DIAGNOSTIC CHARACTERS. The adult male of *K. lebetiformis koreana* **subsp. n.** is separated from *K. lebetiformis lebetiformis* mainly in the features of hypopygium, namely anal point like small rounded protuberance, anal-lateral corners of tergite IX not or slightly elongated (Fig. 9), while male of *K. lebetiformis lebetiformis* with round triangular dark anal point and anal lateral corners of tergite IX elongated as narrow lobes to back (Figs 6–8). A more detailed comparative characteristic of these subspecies is given in Table 1, as well as in the results of DNA barcoding (Fig. 10).

DISTRIBUTION. This subspecies is known only from South Korea.

ETYMOLOGY. The subspecies is named as *koreana* after the type locality in South Korea.

RESULTS OF DNA BARCODING

Genetic variability within the 658 base pair barcode region of COI was examined for 2 individuals belonging to subspecies *Kaluginia lebetiformis lebetiformis*. Both sequences were identical and belonged to the same haplotype. Intersubspecific sequence divergence (K2P) between *K. lebetiformis lebetiformis* and *K. lebetiformis koreana* **subsp. n.** was 0.641. This value is the lower threshold for the species delimitation according to Montagna *et al.* (2016), based on genus *Diamesa* or appropriate threshold in comparison with genera *Polypedilum* (Song *et al.*, 2017) and *Tanytarsus* (Lin *et al.*, 2015). However, according to morphological data (see above), these taxa don't have enough diagnostic features to confirm the species status.

Table 1. Comparison of adult males of *Kaluginia lebetiformis lebetiformis* Makarchenko and *K. lebetiformis koreana* **subsp. n.**

Characters	<i>K. lebetiformis lebetiformis</i> (n=4). South Sakhalin, Amur River basin	<i>K. lebetiformis koreana</i> subsp. n. (n=4). South Korea
Total length, mm	3.2–3.7	2.3–2.6
Wing length, mm	2.28–4.2	2.08–2.52
Total length/wing length	0.82–1.62	1.03–1.01
Head width/palp length	0.84–1.11	0.92–1.0
AR	0.31–0.38	0.34–0.46
Anteprenotals	12–26	18–20
Acrostichals	17–31	25
Dorsocentrals	18–26	21–27
Prealars	17–27	16
Scutellars	73–78	ca 50
Pseudospur number on t_2ta_1	6	4
Tergite IX	With round triangular dark anal point; anal lateral corners elongated as narrow lobes to back.	With anal point like light small rounded protuberance; anal-lateral corners not or slightly elongated.
Transverse sternapodeme length, μm	96–128	128–132
HR	1.74–1.79	1.97

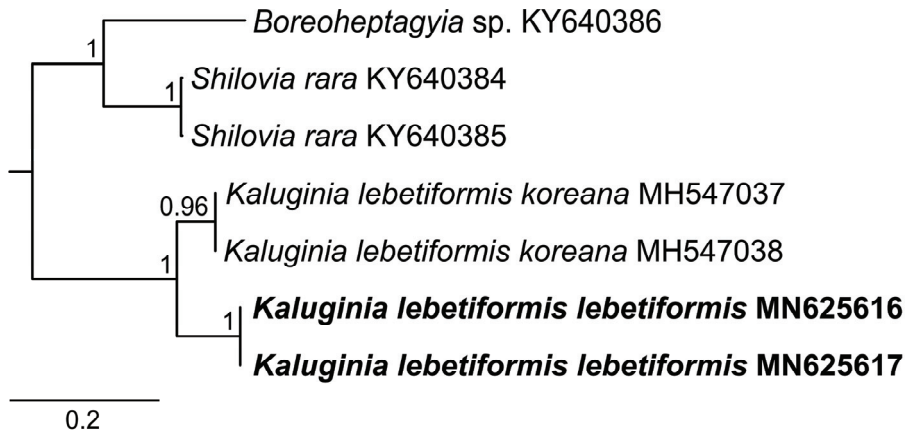


Fig. 10. Bayesian COI mtDNA (658 bp) phylogeny of the tribe Boreoheptagyini from 7 samples, using the SYM + I (1 codon position), F81 (2 codon position) and HKY + G (3 codon position) models of nucleotide substitution. Bayesian posterior probabilities (PP) given in tree nodes. Specimens obtained in this study marked in bold.

We used Bayesian phylogenetic analysis to present relationships of tribe Boreoheptagyiini including *Kaluginia* Makarchenko, *Boreoheptagvia* Brundin and *Shilovia* Makarchenko genera. The BI phylogeny revealed two sister clades, the first consisted of genera *Boreoheptagvia* and *Shilovia* (PP = 1) and the second included two monophyletic *Kaluginia* subspecies (PP = 1) (Fig. 10).

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